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Ankyrins: roles in synaptic biology and pathology

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Abstract

Ankyrins are broadly expressed adaptors that organize diverse membrane proteins into specialized domains and link them to the sub-membranous cytoskeleton. In neurons, ankyrins are known to have essential roles in organizing the axon initial segment and nodes of Ranvier. However, recent studies have revealed novel functions for ankyrins at synapses, where they organize and stabilize neurotransmitter receptors, modulate dendritic spine morphology and control adhesion to the presynaptic site. Ankyrin genes have also been highly associated with a range of neurodevelopmental and psychiatric diseases, including bipolar disorder, schizophrenia and autism, which all demonstrate overlap in their genetics, mechanisms and phenotypes. This review discusses the novel synaptic functions of ankyrin proteins in neurons, and places these exciting findings in the context of *ANK* genes as key neuropsychiatric disorder risk-factors.

Introduction

Ankyrins are a broadly expressed multi-gene family of scaffolding adaptor proteins that have the common function of linking a variety of membrane proteins to the spectrin-based sub-membranous cytoskeleton (Figure 1A). As they primarily organize and stabilize protein networks, they are considered ‘master-organizers’ of membrane-associated protein complexes, and form membrane microdomains throughout the plasma membrane of many different cell types, thereby fulfilling numerous crucial cell biological roles including organization of transverse tubules in cardiomyocytes (Mohler et al., 2003; Mohler et al., 2004) and assembly of the epithelial lateral membrane (Kizhatil and Bennett, 2004; Kizhatil et al., 2007). In neurons, ankyrins play a significant role in organizing the axon initial segment (AIS), the proximal region of the axon which is responsible for neuron firing, as well as the nodes of Ranvier (NoR), sites of action potential regeneration in myelinated

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axons (for a review see (Nelson and Jenkins, 2017)). In addition, recent studies have revealed novel functions for ankyrins at synapses, specialized micron-sized contact-sites between neurons, which require precise organization and regulation to control synaptic transmission. Within excitatory synapses, ankyrins have been shown to arrange and stabilize neurotransmitter receptors, modulate dendritic spine morphology and control adhesion to the presynaptic site. Ankyrin genes have also been highly associated with a range of neurodevelopmental and psychiatric diseases, including bipolar disorder, schizophrenia and autism, all of which demonstrate overlap in their genetics, mechanisms and disease phenotypes. These significant links with human disease and the broad conserved functions of the ankyrin protein family across cell biology has ignited much interest in the potential synaptic functions of ankyrins. Here we collate the most recent work concerning ankyrin function at a variety of synapses, and place it in the context of what is known about ankyrin proteins in other cell-types, and discuss how ankyrins are linked to psychiatric disease, in particular, bipolar disorder.

Ankyrin proteins

The ankyrin protein family

Vertebrate ankyrins comprise of three family members: ankyrin-R, ankyrin-B and ankyrin-G (encoded by *ANK1*, *ANK2* and *ANK3* respectively). The first isoform to be characterized was Ankyrin-R, a scaffolding protein necessary for erythrocyte shape and function, and in addition to its expression in the heart, exhibits a restricted distribution in striated muscle and some neurons in the central nervous system (CNS) (Lambert and Bennett, 1993). Ankyrin-B has a varied expression profile, and is found in tissues including the brain, thymus, heart and skeletal muscle (Cunha and Mohler, 2008; Kunimoto, 1995; Kunimoto et al., 1991; Tse et al., 1991). Ankyrin-B plays a critical role in the heart where it is responsible for the correct localization of ion channels and transporters that are essential for proper calcium signaling and heart function (Mohler et al., 2003). Indeed, a loss-of-function mutation in ankyrin-B, leading to reduced expression, causes type 4 Long QT syndrome, a heart rhythm disorder characterized by erratic heartbeats and lethal cardiac arrhythmias (Mohler et al., 2003). Ankyrin-B is also commonly coexpressed with ankyrin-G in many cell types, including neurons, although they appear to have distinct functions (Abdi et al., 2006; Galiano et al., 2012; Lorenzo et al., 2014; Mohler et al., 2002). In neurons, the 440kDa isoform of ankyrin-B resides in unmyelinated axons where it is though to contribute to an intracellular barrier via interactions with αII and βII spectrin, and thereby limit ankyrin-G localization to the proximal axon (Galiano et al., 2012). The 220kDa variant of ankyrin-B, which is more prominent later in brain development, is shown to be important for long-range axonal transport by coupling the dynein-dynactin motor complex to organelle cargoes such as synaptic vesicles and mitochondria (Lorenzo et al., 2014). Interestingly, knock-out of ankyrin-B causes no AIS abnormalities, suggesting that the roles of the different isoforms of ankyrin-B in the axon are complicated, and require further study(Lorenzo et al., 2014). Compared to its function in the axon little is known about ankyrin-B at mammalian synapses.

Multiple isoforms of ankyrin-G are encoded by *ANK3*, and they are broadly expressed in almost all tissues including the brain, epithelium, kidney and muscle (Devarajan et al., 1996; Kordeli et al., 1995; Peters et al., 1995; Thevananther et al., 1998). Most of the work concerning the myriad functions of ankyrin proteins in the brain have focused on ankyrin-G, therefore this review will focus on these studies with the view of presenting potential models for the roles of ankyrins at synapses, which might also be attributed to ankyrin-B. Multiple isoforms of ankyrin-G exist in neurons, however the 190, 270 and 480kDa isoforms are the most prominent in brain ((Zhang and Bennett, 1998) Figure 1B). The giant 270/480kDa isoforms have well-documented roles as membrane protein and synapse organizers at the AIS and NoR (Leterrier and Dargent, 2014; Nelson and Jenkins, 2017), as well as the 480kDa ankyrin-G having an important role for maintaining GABAergic synapses (Tseng et al., 2015). The role of the 190kDa isoform in neurons is less well characterized, however, evidence now suggests that 190kDa ankyrin-G is an important component of rodent post-synaptic densities and a crucial player in synaptic plasticity (Nanavati et al., 2011; Smith et al., 2014). In addition to the large forms of ankyrin-G, there are also smaller isoforms (110, 116 and 120 kDa), which are localized to the golgi apparatus, endosomes and late endosomes in non-neuronal cells (Devarajan et al., 1996; Hoock et al., 1997; Ignatiuk et al., 2006). Little is known about these isoforms in neurons, however, and combined with the function of ankyrin-B in axonal trafficking described above, these smaller trafficking isoforms indicate that ankyrins (both –B and –G) may play important roles in protein trafficking in neurons, and potentially at synaptic sites.

Ankyrin protein topology

All ankyrin proteins share a common molecular organization with essentially three key functional domains: membrane binding, spectrin binding and regulatory domains (Figure 1B). Ankyrins interact with membrane proteins through their membrane-binding domain which is comprised of 24 N-terminal ANK repeats that are folded into a super-helical solenoid structure (Bennett and Lorenzo, 2013). This region binds an array of highly diverse membrane targets including ion channels, transporters, adhesion molecules, signaling proteins and cytoskeletal elements (Cunha and Mohler, 2009), and provides a platform at the plasma membrane at which these proteins can be arranged. Importantly, the membrane binding regions of all three main ankyrin-G transcripts harbor a cysteine residue (C70), which can be palmitoylated, a process that is required for ankyrin-G association with the membrane, appropriate cellular localization and function (He et al., 2014; He et al., 2012). Palmitoylation of synaptic proteins such as PSD-95 and AMPARs is a key regulator of synaptic function and plasticity (Fukata and Fukata, 2010). It will therefore be intriguing to assess how palmitoylation might regulate the localization and function of ankyrin-G at glutamatergic synapses.

The ankyrin-associated membrane-protein complex interacts with the spectrin/actin cytoskeleton via the spectrin binding domain, through the 160 amino acid ZU5 motif (Mohler et al., 2004; Wang et al., 2012). This motif has conserved sites, such as DAR999 and S2417, which are essential for spectrin binding in the smaller and larger isoforms of ankyrin-G respectively (Jenkins et al., 2015; Kizhatil et al., 2007). Interestingly, mutation of DAR999 in ankyrin-G-190 reduces its synaptic targeting and impairs dendritic spine

structure, underlining the importance of this interaction for glutamatergic synapse stability (Smith et al., 2014).

The C-terminal regulatory domain is comprised of a highly conserved ‘death domain’ and unstructured stretch of 300 amino acids, which modulates interactions with the membrane-binding and spectrin-binding domains (Davis et al., 1992; Hall and Bennett, 1987). Indeed, ankyrins can adopt an autoinhibited conformation to modulate their binding to target membrane-proteins (Abdi et al., 2006; Wang et al., 2014). A recent study has elucidated the autoinhibition mechanisms of ankyrin-B and ankyrin-G: both contain three differing autoinhibition segments within their tail regions, which interact with different sites within their membrane-binding domains (Chen et al., 2017). This therefore enables both -B and -G isoforms to autoregulate in differing ways, resulting in diversity of binding partners and hence cellular localization.

Ankyrins at the neuromuscular junction (NMJ)

The earliest studies of ankyrins at synapses were conducted in mammalian skeletal muscle NMJ, and revealed the primary characteristics of ankyrin function: as an organizer of channels and other proteins in specific membrane domains. Studies of the perijunctional membrane of mammalian NMJ showed that ankyrin-G was localized with spectrin in postsynaptic specializations, which were enriched with voltage-gated sodium channels, (Bailey et al., 2003; Flucher and Daniels, 1989; Kordeli et al., 1998; Wood and Slater, 1998). Further insight into the function of ankyrins at synapses was provided by studies of the *Drosophila* NMJ. At the presynaptic site of the NMJ a novel giant brain-specific *ankyrin2* was identified, the loss of which caused synapse disassembly and retraction, disruption of neuronal excitability and NMJ morphology (Koch et al., 2008; Pielage et al., 2008). Giant *ankyrin2* directly binds and organizes microtubules, thereby contributing to the stability of the presynaptic site and active zones. These studies were the first to describe the organization of ankyrin as a lattice structure and propose the property of this lattice as a linker between synaptic membrane proteins and spectrin, and therefore the underlying microtubule cytoskeleton. In addition, inactivating mutations in *ankyrin2* significantly affected NMJ stability, reducing synaptic terminal size by disintegrating the synaptic cytoskeleton and disassembling active zones (Koch et al., 2008).

Ankyrin-dependent mechanisms at the *Drosophila* NMJ also exist to stabilize synapses during development. For instance, *ankyrin2* phosphorylation by CK2 promotes presynaptic organization and stability by linking synaptic cell adhesion molecules to microtubules, therefore providing a novel mechanism to actively control the development and longevity of synapses (Bulat et al., 2014). Another study shows that interactions between *ankyrin2* and the L1-type cell adhesion molecule, neuroglian, are important in controlling the balance of synapse growth and stability at the *Drosophila* NMJ, as well as coordinating pre- and post-synaptic development in the giant fiber CNS synapse (Enneking et al., 2013). Together, the work on ankyrin proteins at the *Drosophila* NMJ shows that ankyrins form a lattice structure that functions to organize and stabilize the pre-synaptic cytoskeleton through interactions with spectrin, which is also important for pre- and post-synaptic development.

Ankyrins at the mammalian inhibitory synapse

Axon initial segment (Axo-axonal connections)

The AIS is a specialized neuronal membrane domain found at the most proximal region of the axon and the site of inhibitory axo-axonal synapses. It harbors high densities of voltage-gated ion channels, scaffolding molecules and cytoskeletal components, and is primarily responsible for action potential initiation and axonal polarity (Nelson and Jenkins, 2017; Rasband, 2010). The 270/480kDa isoforms of ankyrins are highly expressed at the AIS and are considered ‘master organizers’ of this essential structure due to the dependence of multiple AIS-associated proteins on the presence of ankyrin for their clustering (including neurofascin-186 (NF-186), βIV-spectrin and numerous ion channels (Nelson and Jenkins, 2017; Rasband, 2010)). In addition to the organization of the AIS, ankyrins and their binding partners play a key role in the targeting of GABAergic interneuron presynaptic inputs onto the AIS of excitatory neurons (Figure 2A). GABAergic innervation in this region is essential for controlling the excitability, firing frequency and input-output relationship of neurons in numerous brain regions including hippocampus, cortex and cerebellum (Klausberger and Somogyi, 2008; Kole and Stuart, 2012). GABAergic synapses in the CNS are composed of $GABA_A$ receptor ($GABA_AR$) clusters, which are organized by the scaffolding protein gephyrin ((Smith and Kittler, 2010), Figure 2B): this is also the case for $GABA_AR$ clusters at the AIS (Muir and Kittler, 2014). AIS $GABA_ARs$ are predominantly composed of the α2 subunit, which directly binds to gephyrin (Tretter and Moss, 2008), and gephyrin in turn also indirectly associates with NF-186, which is responsible for targeting inhibitory presynaptic boutons to the AIS (Burkarth et al., 2007). Therefore, although they are not thought to directly interact with the inhibitory PSD at the AIS, ankyrins do anchor NF-186 and are therefore likely in close proximity to gephyrin and $GABA_AR$ clusters, and important for their overall stability.

The importance of ankyrin-G at the AIS was originally demonstrated in cerebellar Purkinje cells, which are innervated at the AIS by inhibitory basket cell axons, forming ‘pinceau synapses’. Knock-out of all *ank3* isoforms in mice caused disruption of the subcellular gradient of NF-186 which exists along the AIS-soma axis (Ango et al., 2004; Zhou et al., 1998). NF-186 is imperative for correct localization of inhibitory synapses originating from basket cell axons and in the absence of ankyrin, these synapses are mislocalized, underlying the importance of ankyrins in correct inhibitory synapse clustering at the AIS (Ango et al., 2004; Buttermore et al., 2012). Ankyrin-G has a similar function at the AIS of cortical excitatory neurons, which receive presynaptic inputs from chandelier cells, forming GABAergic cartridge synapses. The organization of these synapses is highly dependent on the presence of ankyrin proteins (Guan and Maness, 2010; Inda et al., 2006; Zhu et al., 2017). *In vivo*, conditional knock-out of ankyrin-G in cortical pyramidal neurons later in development reveals a dramatic loss of AIS cartridge synapses, leading to elevated cortical activity and behavioral phenotypes that are reminiscent of bipolar disorder (Zhu et al., 2017). In addition, a study of the AIS in monkey prefrontal cortex during development suggests that this ankyrin-G-dependent organization of the AIS changes over time, where ankyrin-G and βIV-spectrin define an early postnatal developmental stage of synapse formation and stabilization at the AIS (Cruz et al., 2009). Combined, these studies suggest a

common mechanism by which ankyrin-G organizes adhesion molecules that that subsequently contribute to the subcellular synaptic organization of inhibitory synapses at the AIS and is essential for both the formation and maintenance of these synapses, and thereby correct excitability of the neuron.

Ankyrin-G at somatodendritic inhibitory synapses

GABAergic synapses target not only the AIS, but also the soma and dendrites of neurons (Megias et al., 2001). Until recently, the functions of ankyrin-G have been thought to be restricted to the AIS and NoR in neurons. However, recent studies have suggested important roles for ankyrins at the synapses of neuronal dendrites (Smith et al., 2014; Tseng et al., 2015). Tseng and colleagues recently showed that the giant 480kDa isoform of ankyrin-G is present at inhibitory synapses in the somatodendritic compartment of pyramidal neurons. Here, ankyrin-G forms micron-scale domains which associate with extrasynaptic $GABA_A$Rs, suggesting it is not directly associated with the inhibitory PSD ((Tseng et al., 2015), Figure 2B). Functionally, this pool of ankyrin-G is thought to contribute to the stability of GABAergic synapses through opposition of $GABA_A$R endocytosis, via an interaction with GABARAP, a $GABA_A$R-associated protein (Fig 2B; Tseng 2015). Interestingly, this paper also suggests that the ankyrin-G-GABARAP interaction might be important for targeting inhibitory synapses to the AIS, pointing to potential common mechanisms of GABAergic synapse stability in both membrane regions. Another group also suggested ankyrin-B may contribute to the development and stability of perisomatic and proximal dendritic inhibitory synapses in the mouse cingulate cortex, possibly through an interaction with L1-CAM (Guan and Maness, 2010). Clearly further work is now required to assess how the interactions between these different proteins lead to the stability of inhibitory synapses outside of the AIS and therefore maintain inhibition.

Ankyrins at the PSD of the mammalian glutamatergic synapse

In addition to its functions at somatodendritic inhibitory synapses, it has now become clear that ankyrins also serve to facilitate synaptic function and plasticity. More than a decade ago, proteomic analyses detected all three ankyrins in rodent PSD preparations (Collins et al., 2006; Jordan et al., 2004; Peng et al., 2004). Later analysis of the hippocampal PSD proteome after treatment with the mood stabilizing drugs lithium and valproate showed increased levels of the 190kDa ankyrin-G in synapses (Nanavati et al., 2011), thus linking ankyrin-G and excitatory synapses to psychiatric disease. More recently, our own laboratory used a range of techniques to show that the 190kDa isoform of ankyrin-G is integral for maintenance of spine morphology and glutamatergic synaptic transmission (Smith et al., 2014), and the first to demonstrate a function for ankyrin-G in neurons outside its canonical functions at the AIS and NoR.

A role for Ankyrin-G 190kDa isoform in synapse maintenance

The longer isoforms of ankyrin-G have well-defined functions at the AIS and NoR; however, 190 kDa ankyrin-G has a key role in the maintenance and function of mature cortical synapses (Figure 2C). RNAi knock-down of all ankyrin-G isoforms in cultured cortical neurons causes reduced dendritic spine area, mEPSC amplitude and AMPAR clustering,

indicating that ankyrin-G is important for maintaining the strength of excitatory synapses (Smith et al., 2014). Interestingly, these effects were rescued with an RNAi-resistant form of the 190kDa isoform of ankyrin-G (ankyrin-G-190) but not the longer, 270kDa isoform (ankyrin-G-270), suggesting that ankyrin-G-190 may be mediating these effects. In addition, confocal and super-resolution imaging of ankyrin-G-190 tagged with GFP showed enrichment of this isoform at postsynaptic sites within mature spines, compared with ankyrin-G-270, which was restricted to the AIS. Together, the ability of ankyrin-G-190 isoform to rescue the phenotypes elicited by ankyrin-G RNAi, and the opposing effects that overexpression of this isoform has on spine morphology suggests that ankyrin-G-190 is present in spines and is important for the maintenance of glutamatergic synapse morphology and efficacy.

Ankyrin-G forms nanoscale clusters in dendritic spines

Confocal imaging demonstrated the colocalization of endogenous ankyrin-G with glutamatergic synaptic markers, however higher resolution imaging was required to uncover nanoscale subsynaptic detail that would point to potential functions for ankyrin-G. The advent of super-resolution microscopy methodologies has facilitated imaging beyond the diffraction limit of light, providing unprecedented insight into the nanoscale architecture of synapses (reviewed in detail in (MacGillavry and Hoogenraad, 2015; Tonnesen and Nagerl, 2013)). Indeed, several recent papers have utilized these methods to show the nanoscale organization of AMPARs, PSD95, actin, adhesion molecules and CamKII (Frost et al., 2010; Lu et al., 2014; MacGillavry et al., 2013; Smith et al., 2017; Tang et al., 2016). Smith *et al.* used the versatile super-resolution method Structured Illumination Microscopy (SIM), to image ankyrin-G in dendritic spines, with a cell fill to visualize spine morphology and either immunolabeled PSD95 or GluA1 subunit-containing AMPARs. These imaging experiments revealed that, in contrast a classical scaffold, ankyrin-G forms distinct condensed clusters of ~130nm in diameter, that are organized perisynaptically around the PSD, and within the spine neck ((Smith et al., 2014), Figure 2C).

What are the functions of perisynaptic and spine neck pools of ankyrin-G? As described above, knock-down studies show that ankyrin-G is required for the stability and maintenance of spines and AMPAR-mediated currents by stabilizing AMPARs in the spine. We propose that ankyrin-G performs this task in two ways: (1) as a perisynaptic scaffold and (2) by contributing to a diffusional barrier in the spine neck (Figure 3A). The *perisynaptic* pool of ankyrin-G may be important in the organization of the distinct perisynaptic region which boarders the PSD. This perisynaptic region is thought to be compositionally different from the PSD: metabotropic glutamate receptors (mGluRs, (Lopez-Bendito et al., 2002)), dopamine D1 receptors (Ladepeche et al., 2013) and the endocytic machinery (Blanpied et al., 2002) are thought to localize to this region. Moreover, the perisynaptic region exhibits a highly dynamic pool of continuously polymerizing F-actin (Frost et al., 2010), which contributes to the continual morphological distortion of the PSD and the anchoring of AMPARs. Functionally, the perisynaptic region is thought to control the exchange between synaptic and extrasynaptic glutamate receptors and provide a reservoir of ‘back-up’ glutamate receptors, poised to move into the PSD to potentiate synaptic transmission (Tardin et al., 2003). The position of ankyrin-G in this region, its role as a scaffold linking

membrane proteins to the cytoskeleton and the observation that ankyrin-G complexes with GluA1 from brain extracts, suggests that it may act as a scaffold for perisynaptic AMPARs and other membrane proteins. Therefore, ankyrin-G likely stabilizes this perisynaptic region and ensures an abundance of extra AMPA receptors are readily available. In addition, the direct links between ankyrin-G and the spectrin-actin cytoskeleton at the perisynaptic region and in the spine head as a whole would add structural stability to the spine and therefore promote AMPAR retention at the synapse.

The second mechanism by which ankyrin-G contributes to spine and AMPAR stability is likely through its localization to the spine neck, an essential structure that underlies the biochemical and electrical compartmentalization of excitatory synaptic signals (Tonnesen and Nagerl, 2016). The spine neck functions as a diffusional barrier that restricts molecular exchange between the spine head and dendritic shaft, thereby compartmentalizing synaptic signaling events (Ashby et al., 2006; Kusters et al., 2013; Simon et al., 2014). This is supported by simultaneous analysis of diffusive cytosolic proteins in and out of the spine showing that spine neck width is a significant determinant of the ability of proteins to pass through the spine neck (Takasaki and Sabatini, 2014; Tonnesen et al., 2014). Importantly, spine neck geometry changes with neuronal activity (Bloodgood and Sabatini, 2005; Grunditz et al., 2008; Smith et al., 2014; Takasaki and Sabatini, 2014; Tonnesen et al., 2014). Indeed, remarkable live-cell super-resolution imaging of spines in hippocampal slices revealed that spine neck diameter increases during LTP induced by glutamate-uncaging at the spine head, with important implications for neck resistance and diffusional recovery of the spine (Tonnesen et al., 2014).

Despite its importance in neuronal function, the molecular mechanisms that control spine neck morphology and function remain undefined. To address this dearth of mechanistic knowledge, multiple groups have employed super-resolution microscopy and electron microscopy to aid studies of the spine neck molecular composition. Photoactivated Localization Microscopy (PALM) has been used to visualize dynamically polymerizing F-actin (Frost et al., 2010), immunogold-EM has been utilized to localize septin-7 and βIII-spectrin to the neck region (Efimova et al., 2017; Ewers et al., 2014), and synaptopodin was recently shown to localize with F-actin in the neck (Wang et al., 2016). Using SIM imaging, ankyrin-G nanodomains were observed in the neck of 74% of spines in addition to its presence in the spine head (Smith et al., 2014). Spines with ankyrin-G in the neck were larger contained a higher abundance of AMPARs, but not PSD95, suggesting that neck ankyrin-G plays a role in modulating the synaptic localization of dynamic, diffusive proteins rather than the relatively static PSD itself. Further, the overexpression of ankyrin-G-190 in the spine neck in addition to the head also was associated with larger spine heads and wider spine necks in mature spines (Smith et al., 2014), suggesting that ankyrin-G may play a direct role in modulating spine neck morphology. This is likely due to association with F-actin in this region via an interaction with β-spectrin, which is also abundant in the spine neck (Efimova et al., 2017) and is essential for synaptic function (Nestor et al., 2011). More work is now required, with the assistance of super-resolution microscopy modalities, to understand how proteins that are found in the spine neck work in concert to regulate spine dimensions and generate a diffusional barrier.

Together, in dendritic spines, ankyrin-G contributes to maintaining the synaptic AMPAR complement by their stabilization at the perisynaptic region where they can be readily added to the PSD to potentiate synaptic function. Neck ankyrin-G also likely contributes to a diffusional barrier in the spine neck (akin its function at the AIS), limiting the mobility of AMPARs (and presumably other membrane proteins), preventing their movement out of the spine and thereby maintaining their presence in the spine head.

Ankyrin-G contributes to LTP-dependent spine enlargement

In addition to its importance in the maintenance of glutamatergic synapses, ankyrin-G is essential for spine plasticity during LTP. Knock-down of ankyrin-G in cortical neurons impairs the ability of spines to undergo enlargement during a chemical LTP (cLTP) protocol (Figure 3 (Smith et al., 2014)). Moreover, SIM imaging shows that during cLTP, ankyrin-G nanodomains accumulate in spines and demonstrate greater overlap with AMPARs during LTP (Smith et al., 2014). Considering that overexpression of ankyrin-G in spines causes increased spine area, it is likely that ankyrin-G accumulation in spines during LTP plays a key role in spine enlargement during synaptic plasticity. During this early stage of LTP, actin undergoes rapid polymerization, CamKII is activated, and specific proteins such as AMPARs and actin remodeling proteins are trafficked into the spine head (Sala and Segal, 2014). This, combined with the location of ankyrin-G nanodomains at the spine neck and at the interface of the PSD and the actin cytoskeleton, regulatory sites for synaptic plasticity (Cingolani and Goda, 2008), points to a key role for ankyrin-G in the modulation of spine LTP. The scaffolding role of ankyrin-G also makes it a major candidate for providing more anchoring platforms for multiple membrane proteins including ion channels and NMDARs, which are enriched in the spine during plasticity and are essential for LTP induction and expression.

These studies in cultured cortical neurons suggest an *in vivo* role for ankyrin-G in learning and memory. Support for this comes from analysis of hippocampal lysates from mice that have undergone a learning task: significantly more 190 kDa ankyrin-G was found in the membrane and cytoskeletal fractions of trained mice compared with control mice, indicating that a physiological learning paradigm can cause relocation of a pool of ankyrin-G *in vivo* (Smith et al., 2014). This is also corroborated by a study using *Drosophila* showing that knockdown of *ankyrin2* results in reduced synapse size and number, and behavioral deficits in short-term memory (Iqbal et al., 2013). Further work will now be required to fully determine the role(s) of synaptic ankyrin-G in synaptic plasticity and in learning and memory *in vivo*.

Roles of ankyrins in neurodevelopmental and psychiatric disease

As a key organizer of multiple types of synapses, disruption of ankyrin function and expression might be expected to have detrimental effects on numerous brain functions. Accordingly, the human *ANK3* gene is associated with a variety of neuropsychiatric and cognitive disorders including bipolar disorder (BD), schizophrenia, autism spectrum disorder (ASD), intellectual disability (ID) and ADHD (Ferreira et al., 2008; Schulze et al., 2009; Scott et al., 2009; Smith et al., 2009). The role that alterations in *ANK3* play in the

pathogenesis of these disorders remains elusive, however, disrupted synaptic function (either at the AIS, inhibitory or excitatory synapses) is a likely mechanism due to the convergence of synaptic pathology as an underlying mechanism for these disorders (Forrest et al., 2018; Penzes et al., 2011). Indeed, function of ankyrin-G as a synaptic scaffold, anchoring AMPA receptors, $GABA_A$Rs and a plethora of ion channels at important neuronal contact sites, suggests that its disruption would cause myriad alterations to the excitatory/inhibitory balance in neurons and circuits, which is likely to lead to alterations in information processing, cognition and behavior (Gao and Penzes, 2015; Yizhar et al., 2011). Indeed, the fact that mutations and polymorphisms in *ANK3* link this gene to a wide range of mental disorders, point to both how critical ankyrin-G is to neuronal function, and how disruption in its expression can lead to a variety of overlapping behavioral and cognitive characteristics

Multiple independent genome wide association studies (GWAS) have strongly linked *ANK3* to bipolar disorder (Baum et al., 2008; Ferreira et al., 2008; Schulze et al., 2009; Tesli et al., 2011), making it one of the leading BD risk genes. These association studies have been replicated in a variety of different types and sizes of populations, thereby strongly supporting the association between BD and *ANK3* (Muhleisen et al., 2014; Roby, 2017; Scott et al., 2009). Furthermore, genome sequencing has revealed BD-associated rare variants in *ANK3* (Ament et al., 2015; Hughes et al., 2016), although how they affect ankyrin-G expression and function remains to be determined. The causality of BD has a large genetic component, with heritability estimated at 59–93% (Kieseppa et al., 2004; Lichtenstein et al., 2009; McGuffin et al., 2003), however the pathogenesis of BD is poorly understood, exemplified by the continued use of the mood-stabilizer, lithium, to treat BD patients. Interestingly, the expression of the 190kDa isoform in rat hippocampal PSDs increases after treatment with lithium, underscoring the relevance of ankyrin-G function in BD brains (Nanavati et al., 2011).

Although, primarily thought of as a BD-associated gene, *ANK3* is also genetically linked to multiple other neuropsychiatric disorders. GWAS and meta-analysis have identified *ANK3* as an important schizophrenia risk factor (Nie et al., 2015; Schizophrenia Psychiatric Genome-Wide Association Study, 2011)). Moreover, analysis of post-mortem brain tissue from schizophrenic patients reveal a 15–19% loss of ankyrin-G expression in the AIS of pyramidal neurons in the superficial cortex compared to controls (Cruz et al., 2009). Rare pathogenic mutations in *ANK3* have been identified in patients with ID/ADHD (Bonnet-Brilhault et al., 2016; Iqbal et al., 2013; Kloth et al., 2017) and ASD (Bi et al., 2012; Shi et al., 2013). Inactivating mutations in the *ANK3* gene result in severe cognitive deficit. A balanced translocation, that disrupts expression of all *ANK3* isoforms, was identified in a patient with cognitive deficits, ADHD and ASD (Iqbal et al., 2013). Further, the same study reported the first familial mutation in *ANK3*, identified in a family with autosomal recessive ID and severe behavioral problems (Iqbal et al., 2013). This mutation produced a premature stop codon in the 480 kDa ankyrin-G isoform, leading to severely reduced expression. Together, the identification of *ANK3* disruption in patients with these disorders supports the functions of ankyrin-G at synapses and the importance of ankyrin-G in cognitive function in humans.

Modeling BD with Ankyrin-G KO mice

Several mouse models of ankyrin-G depletion have been generated and characterized, showing a set of psychiatric-related behaviors consistent with *ANK3* involvement in neuropsychiatric disorders and the functions of ankyrin-G at synaptic sites. Ablation of brain-specific *Ank3* isoforms in the cerebellum causes early onset ataxia, due to impaired action potential firing at the AIS of purkinje cells in the cerebellum (Zhou et al., 1998). In comparison, heterozygous knock out of brain-specific *Ank3* isoforms in mice results in altered mood related behaviors such as reduced anxiety and increased reward motivation. Interestingly, the behavioral traits of *Ank*3+/− mice transitioned to depression-related features after chronic stress, a trigger of mood episodes in BD (Leussis et al., 2013). In addition, viral-mediated RNA interference of *Ank3* expression in the hippocampal dentate gyrus also induced decreased anxiety-related behaviors and increased activity, which were attenuated by chronic treatment with the mood stabilizer lithium (Leussis et al., 2013). This finding was replicated in another study (Gottschalk et al., 2017), in which the authors used proteomics of *Ank3*+/− mice treated with lithium to analyze changes in protein levels with the view of determining mechanism of action for this drug. This revealed that axonal transport and the kinesin family of motor proteins may be important for the function of lithium in *Ank3*+/− mice, in concert with changes to the glutamatergic signaling system. Further evidence linking ankyrin-G function to BD-like behavior was recently provided through study of a conditional *Ank3* KO mouse, where *Ank3* expression was reduced in pyramidal neurons of the adult forebrain (Zhu et al., 2017). This resulted in loss of pyramidal neuron AIS voltage-gated sodium and potassium channels, loss of GABAergic cartridge synapses, and increased c-fos expression suggesting increased neuronal activity due to disinhibition. Importantly, these mice showed behavioral phenotypes reminiscent of aspects of human mania, ameliorated by lithium and valproate (Zhu et al., 2017). These behavioral deficits have been further replicated further in heterozygous KO mice that display elevated anxiety, depression and cognitive impairment, combined with reduced size of various brain regions including hippocampus and motor cortex (Liu et al., 2017; van der Werf et al., 2017).

Given the important role of synapse dysfunction in these neuropsychiatric disorders, it is therefore tempting to speculate that at least some of these phenotypes are caused by ankyrin-mediated dysfunction of synapses, alterations to E/I balance and disrupted circuitry in critical forebrain regions. However, additional work will be required to determine the underlying cellular mechanisms, and which isoforms of ankyrin-G, at which types of synapses contribute to specific behavioral phenotypes.

Conclusions and future challenges

The study of ankyrins at dendritic synapses is still in its infancy, however, common mechanisms of ankyrin function at all types of synapses have emerged, providing essential maintenance of synapse structure and function. It is clear that ankyrins form micro/nanodomains in specific regions of the NMJ, and at excitatory and inhibitory PSDs. Furthermore, a membrane protein-ankyrin-spectrin complex forms a lattice under the plasma membrane, where it can both anchor synaptic membrane proteins, in addition to intracellular

binding-partners. Although there is now an outline for ankyrin function at synapses outside of the AIS, there are significant gaps in our knowledge leading to many unanswered questions. For instance, what other glutamatergic and GABAergic synaptic proteins does ankyrin-G complex with? Similarly to at the AIS, it is likely that ankyrin-G interacts with numerous receptors, channels, adhesion proteins and signaling molecules in dendritic spines and at somatodendritic inhibitory synapses. Identification of these binding-partners will provide us with a greater understanding of the role that ankyrin-G plays at these synapses and its involvement in mechanisms that mediate synaptic function and plasticity.

Are other ankyrins present at CNS synapses? Proteomic analysis suggests that all three ankyrin proteins (-R, -B and -G) are expressed in glutamatergic PSDs (Collins et al., 2006; Jordan et al., 2004; Peng et al., 2004), suggesting that in addition to ankyrin-G, ankyrin-R and ankyrin-B may also have important functions in dendritic spines. Determining cell-type and synapse-type abundance of these ankyrins will be paramount in understanding how these proteins work together to maintain synapse strength and contribute to synaptic plasticity. Additionally, little is known about ankyrin function at glutamatergic or GABAergic presynaptic terminals. At the NMJ, ankyrin-B functions to create a lattice network around which the presynaptic machinery can be organized (Koch et al., 2008; Pielage et al., 2008). Further, ankyrin-G localizes with the excitatory and inhibitory presynaptic marker, bassoon (Smith et al., 2014), therefore it is possible that ankyrins may have a similar functions at mammalian CNS synapses, making them essential for presynaptic function and therefore synaptic transmission. Finally, a role for ankyrins in protein trafficking described in non-neuronal cells (Devarajan et al., 1996; Hoock et al., 1997) and axonal transport (Barry et al., 2014; Lorenzo et al., 2014) also opens up new avenues of research, and intriguing possibilities for ankyrin function in trafficking of key proteins to and from the synapse. These represent just a handful of potential interesting future directions with the aim of fully understanding how ankyrins contribute to synapse function and plasticity. Importantly, future work focused on revealing the mechanisms of ankyrin function at synapses will likely provide insight into novel therapy targets for neuropsychiatric disorders.

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Highlights

- Ankyrin proteins link membrane proteins to the submembranous actin cytoskeleton
- Ankyrins organize synapses, in addition to their canonical roles in the axon
- Dendritic spines rely on ankyrin-G for proper stability and synaptic plasticity
- Synaptic functions of ankyrins may contribute to neuropsychiatric diseases

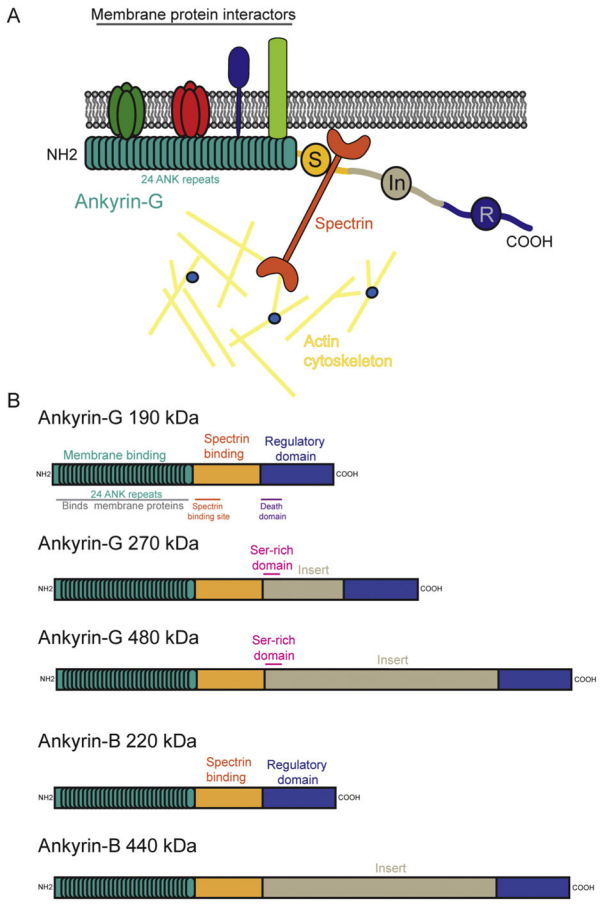

Figure 1. Ankyrins: function and domain structure

A. Ankyrins have a conserved function across many cell types; to link membrane proteins to the submembranous actin-spectrin cytoskeleton.

B. The domain structure of ankyrin-G and ankyrin-B, the main ankyrins to have characterized functions at synapses. The domain structure is conserved across all ankyrins. 24 ankyrin repeats form the membrane binding domain which is the binding site for membrane targets that are organized by ankyrins. Ankyrins interact with the actin-spectrin cytoskeleton via the spectrin-binding region (S), and the c-terminal regulatory domain is responsible for modulating the interactions of the membrane binding domain. Larger ankyrin isoforms have a neuronal specific insert. Ankyrin-G 270/480 kDa harbor a serine-rich domain which is responsible for targeting these isoforms to the AIS.

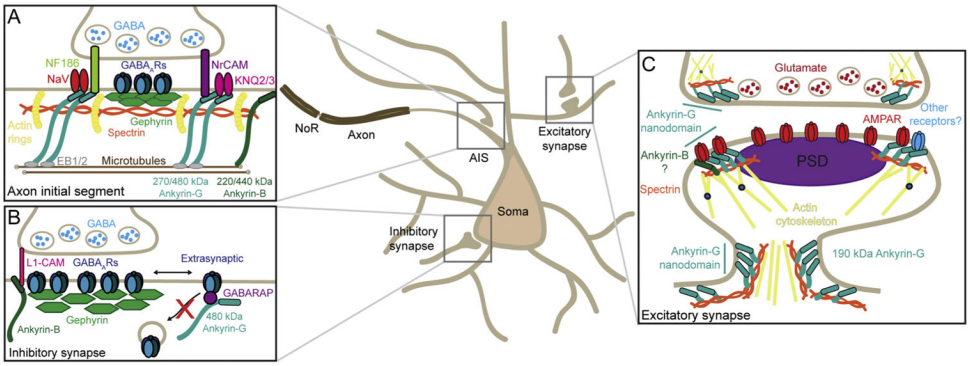

Figure 2. Ankyrin functions at synapses

A. Ankyrins at axo-axonic synapses at the axon initial segment (AIS). Larger isoforms of ankyrin-G and ankyrin-B are found in myelinated and unmyelinated axons respectively. Ankyrin-G scaffolds ion channels and cell adhesion molecules, the latter of which recruit presynaptic terminals of interneurons to the AIS.

B. Ankyrins at somato-dendritic inhibitory synapses. The giant 480 kDa isoform of ankyrin-G is localized to the extrasynaptic regions of somato-dendritic inhibitory synapses. Here it also functions to stabilize the synapse through an interaction with the $GABA_AR$ associated protein (GABARAP), whereby it opposes the endocytosis of extrasynaptic $GABA_ARs$. Ankyrin-B is also thought to contribute to stability of perisomatic and proximal dendritic inhibitory synapses through L1-CAM.

C. Ankyrins at the dendritic spine. Ankyrin-G forms nanodomains at perisynaptic regions and in the spine neck, where it interacts with spectrin/actin cytoskeleton to modulate spine head and neck dimensions. Ankyrin-G interacts with AMPA receptors (AMPARs) and promotes receptor stability at the synaptic site. Ankyrin-G is also localized at the presynaptic terminals of glutamatergic synapses of CNS neurons, and of the neuromuscular junction.

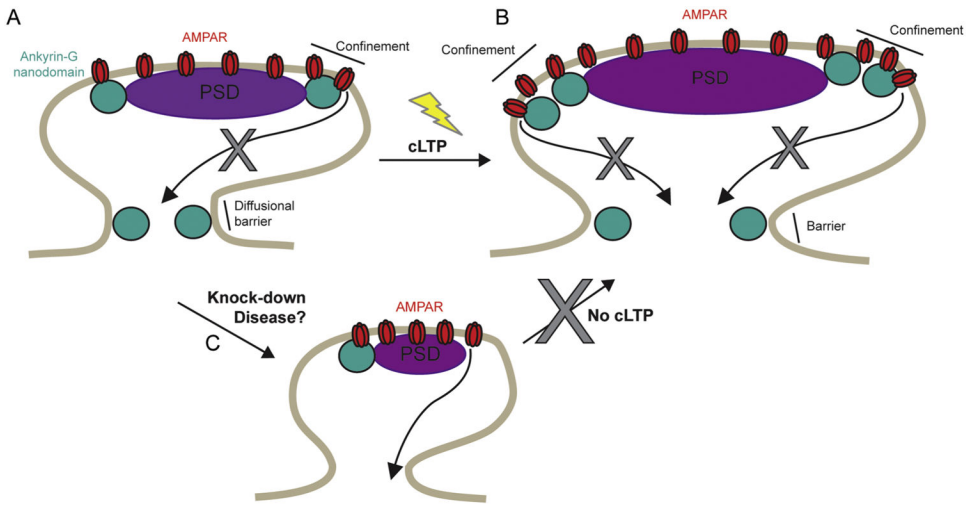

Figure 3. A role for ankyrin-G in synaptic plasticity

A. In dendritic spines ankyrin-G functions to confine AMPA receptors (AMPARs) and stabilize them at the PSD. **B.** During chemical LTP stimuli, more ankyrin-G moves into the spine where it further stabilizes the PSD and AMPARS, and likely contributes to structural enlargement and stability of the spine through its interactions with the actin-spectrin cytoskeleton. **C.** Knock-down of ankyrin-G causes decreased spine area and glutamatergic synaptic transmission, and makes spines unable to undergo cLTP-dependent spine enlargement.